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Of Mice and MEN1: Insulinomas in a Conditional Mouse Knockout

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Patients with multiple endocrine neoplasia type 1 (MEN1) develop multiple endocrine tumors, primarily affecting the parathyroid, pituitary, and endocrine pancreas, due to the inactivation of the *MEN1* gene. A conditional mouse model was developed to evaluate the loss of the mouse homolog, *Men1*, in the pancreatic beta cell. *Men1* in these mice contains exons 3 to 8 flanked by loxP sites, such that, when the mice are crossed to transgenic mice expressing cre from the rat insulin promoter (RIP-cre), exons 3 to 8 are deleted in beta cells. By 60 weeks of age, >80% of mice homozygous for the floxed *Men1* gene and expressing RIP-cre develop multiple pancreatic islet adenomas. The formation of adenomas results in elevated serum insulin levels and decreased blood glucose levels. The delay in tumor appearance, even with early loss of both copies of *Men1*, implies that additional somatic events are required for adenoma formation in beta cells. Comparative genomic hybridization of beta cell tumor DNA from these mice reveals duplication of chromosome 11, potentially revealing regions of interest with respect to tumorigenesis.

Multiple endocrine neoplasia type 1 (MEN1; Online Mendelian Inheritance in Man no. 131100) is a familial tumor syndrome characterized primarily by multiple endocrine tumors of the parathyroids, anterior pituitary, and pancreas. The gene responsible for MEN1, located on chromosome 11q13 (21), was identified in 1997 by positional cloning (5), and germ line mutations in *MEN1* have been found in the majority of MEN1 kindreds (1, 4, 27). Somatic *MEN1* mutations in sporadic parathyroid adenomas, pituitary tumors, insulinomas, gastrinomas and lung carcinoids have also been reported (7, 9, 16, 33, 34). The protein product of *MEN1*, termed menin, is ubiquitously expressed, is targeted to the nucleus (13), and has been reported to interact with a variety of proteins, including JunD (2), Smad3 (19), Pem (22), Nm23 (25), NF-κB (15), and RPA2 (32).

The mouse *Men1* gene has also been characterized (12, 31), and the protein product has been found to be 97% identical to human menin. As with many tumor suppressor gene knockouts, homozygous *Men1* knockout mice die in utero between embryonic days 11.5 and 12.5. These mice exhibit delayed development and craniofacial abnormalities, perhaps associated with neural tube closure defects (6). This lethality has made it impossible to study the tumorigenic effects following germ line loss of both *Men1* alleles. Heterozygote *Men1* knockout mice develop an endocrine tumor spectrum similar to the human MEN1 phenotype, including pancreatic, pituitary, and parathyroid lesions (6).

To overcome the early lethality of homozygotes and to study the function of *Men1* in both the developing mouse pancreas and during tumorigenesis, we engineered specific deletion of exons 3 to 8 of the *Men1* gene in the pancreatic beta cells using the cre-loxP system. Mice in which exons 3 to 8 of *Men1* were flanked by loxP sites (floxed) were generated. Exons 3 to 8 were then deleted by breeding the mice with one of three independent lines of transgenic mice expressing cre from the rat insulin promoter (RIP-cre). In the homozygous state, this deletion leads to adenoma formation. Mice with conditional *Men1* deletions develop elevated insulin levels and decreased blood glucose levels, which correlate with the onset of pancreatic beta cell tumors. These beta cell tumors express insulin and demonstrate increased proliferation, as shown by bromodeoxyuridine (BrdU) staining.

MATERIALS AND METHODS

Gene targeting and genotyping. Floxed *Men1* mice were generated by breeding the existing line $Men1^{TSM/+}$ (6) with EIIa-cre transgenic mice, which express cre ubiquitously from the EIIa promoter (20). The resulting $Men1^{\Delta N/\Delta N}$ mice were of mixed FVB;129Sv background and contained loxP sites in introns 2 and 8 of *Men1*. Only $Men1^{\Delta N/\Delta N}$ mice without EIIa-cre present were used in subsequent breedings to RIP-cre transgenic lines. Breeding $Men1^{\Delta N/\Delta N}$ mice to RIP-cre lines resulted in mixed B6;FVB;129Sv backgrounds. To avoid aberrant results from the genetic background, progeny from breeding $Men1^{\Delta N/\Delta N}$ mice with each of the three RIP-cre lines were compared only to littermates from the same breedings. The full strain designation of RIP2-cre is C57BL/6-TgN(Ins2Cre)25Mgn.

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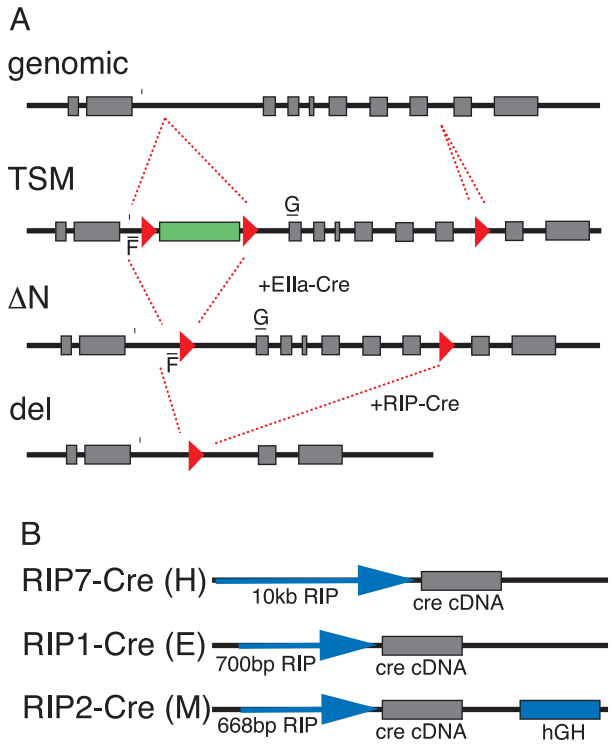


FIG. 1. Genomic structure of *Men1* alleles and RIP-cre transgenes. (A) In vivo manipulation of the *Men1* gene. The genomic structure of the *Men1* gene (top line) was altered by the insertion of a floxed 3-phosphoglycerate kinase (PGK)–neomycin cassette in intron 2 and a third loxP in intron 8 to generate the TSM allele in $Men1^{TSM/+}$ mice, as previously reported (6). $Men1^{TSM/+}$ mice were then bred to EIIa-cre transgenic mice, and the progeny were selected for loss of the PGK-neomycin cassette; the resulting allele was termed ΔN. Breeding $Men1^{\Delta N/\Delta N}$ mice to RIP-cre transgenic mice resulted in progeny with exons 3 to 8 excised in a tissue-specific manner to generate the del allele. Grey boxes, exons; red triangles, loxP sites; green box, PGK-neomycin cassette in the transcriptional orientation opposite to that for *Men1*; small black bars, genotyping primer locations F and G. (B) RIP-cre transgenes. Three different lines of cre-expressing mice were utilized, each with the cre recombinase under the control of a portion of the rat insulin promoter (RIP).

All mice were genotyped by PCR for the various cre transgenes with primers 5′cre (5′-CCGGGCTGCCACGACCAA) and 3′cre (5′-GGCGCGGCAACAC CATTTTT). $Men1^{\Delta N/\Delta N}$ mice were genotyped with primers F (5′-GCCATTTC ATTACCTCTTTCTCCG) and G (5′-TACCACTGCAAAGGCCACGC) to demonstrate loss of the floxed neomycin cassette. All mice were maintained in accordance with National Institutes of Health and Association for the Assessment and Accreditation of Laboratory Animal Care guidelines.

Histochemistry. Staining for lacZ and alkaline phosphatase (AP) was performed as previously reported (23) with minor modifications. Briefly, tissue for AP staining was removed, flash frozen in isopentane over liquid nitrogen, and then embedded in OCT freezing medium. Eight-micrometer sections were fixed in 0.2% glutaraldehyde and then washed in phosphate-buffered saline (PBS) prior to inactivation of the endogenous AP by incubation at 70°C in PBS. Tissue sections were then stained in nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) overnight, washed in PBS, dehydrated, and sealed under coverslips. Tissue for lacZ staining was flash frozen, sectioned, and fixed as described above and then stained for 3 to 6 h in lacZ stain, washed in PBS, dehydrated, and mounted under coverslips.

IHC. (i) Hormone IHC. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4 to 6 μm. For some immunohistochemistry (IHC) experiments, sections were pretreated in water in a microwave oven (11). A guinea pig polyclonal antibody against porcine pancreatic insulin was used at a 1:500 dilution for insulin, a rabbit antiprolactin antibody was used at 1:2,000, and rabbit antiadrenocorticotropin and rabbit antiglucagon were used at 1:1,000 (all antibodies were from DAKO, Carpinteria, Calif.). The Vectastain ABC guinea pig or rabbit kit (Vector Laboratories, Burlingame, Calif.) was used with diaminobenzidine (DAB) as the chromogen.

(ii) Menin IHC. Tissues were fixed in 4% paraformaldehyde, washed in PBS, embedded in paraffin, and sectioned at 4 to 6 μm. Slides were pretreated by microwaving in 10 mM citric acid buffer, pH 6.0, prior to treatment with 30% hydrogen peroxide to quench endogenous peroxidases. Slides were then blocked with avidin D (Vector Laboratories), biotin (Vector Laboratories), and protein blocking agent (Fisher Scientific, Pittsburgh, Pa.) prior to staining with primary antimenin rabbit polyclonal antibody SQV-R4 or SQV-R5 at a 1:500 dilution (13). A secondary antibody from the Vecta Elite kit (Vector Laboratories) was used with DAB as the chromogen. Slides were counterstained with Gill's hematoxylin (Fisher Scientific).

Immunofluorescence. Slides were treated as for menin IHC described above, except that primary antibody incubation included a combination of antimenin SQV-R4 or SQV-R5 at a 1:500 dilution and either guinea pig anti-insulin (DAKO) at 1:400 or guinea pig antiglucagon (Linco, St. Charles, Mo.) at 1:100. Secondary antibodies were a combination of Cy3-conjugated donkey anti-rabbit and Cy2-conjugated donkey anti-guinea pig (Jackson Immunoresearch, West Grove, Pa.), both at 1:300 dilution. Autofluorescence was suppressed by incubation for 10 min in 0.2% Sudan Black B solution in 70% ethanol prior to washing in water and mounting in fluorescent mounting medium (Kirkegaard & Perry Laboratories, Gaithersburg, Md.).

Islet morphometry. Four-micrometer sections of pancreas were stained with a combination of anti-insulin and antiglucagon antisera as indicated above to identify islets in *RIP-cre* and $Men1^{\Delta N/\Delta N}$; *RIP-cre* animals. Three or four sections separated by 300 μm were analyzed for each animal. Islets were counted, and the area was measured with IPLab (Fairfax, Va.) software. Average radii were calculated from area measurements and then used to calculate average volume under the assumption that islets are spherical.

Blood chemistry. Blood specimens were collected from all mice by retro-orbital sampling. Blood glucose was measured with a Glucometer Elite II (Bayer, Elkhardt, Ind.) on whole blood at the time of collection. Insulin levels were determined by radioimmunoassays (Linco). All mice were fasted for 8 h prior to phlebotomy.

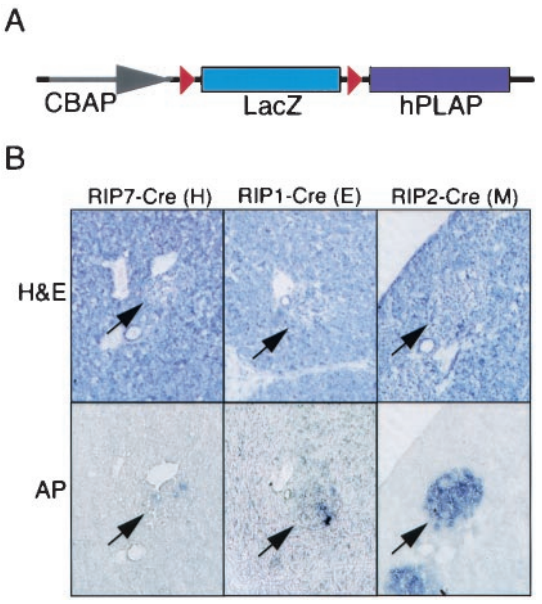


FIG. 2. Expression pattern of Cre in pancreata of RIP-cre transgenic mice bred to Z/AP reporter mice. (A) Structure of the Z/AP reporter transgene. Z/AP reporter mice contain the chicken beta actin promoter (CBAP) followed by a floxed lacZ gene and then a heat-stable human placental AP (hPLAP) gene (23). Red triangles, loxP sites. (B) All three lines of RIP-cre mice were bred to transgenic Z/AP mice. The pancreata of 6-week-old mice carrying both transgenes were serially sectioned, and neighboring sections were stained with either hematoxylin and eosin (H&E) or AP. Arrows, pancreatic islets.

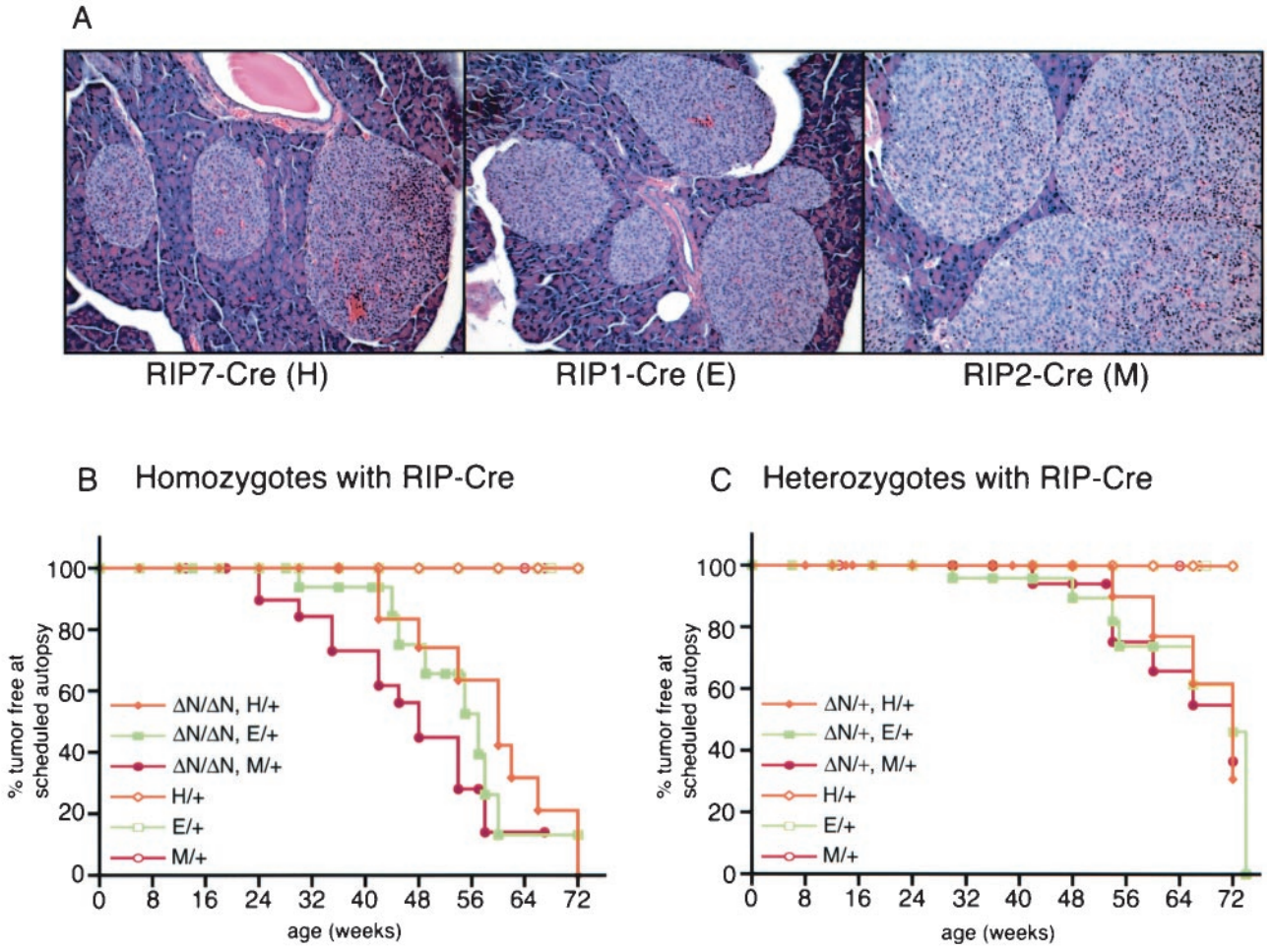


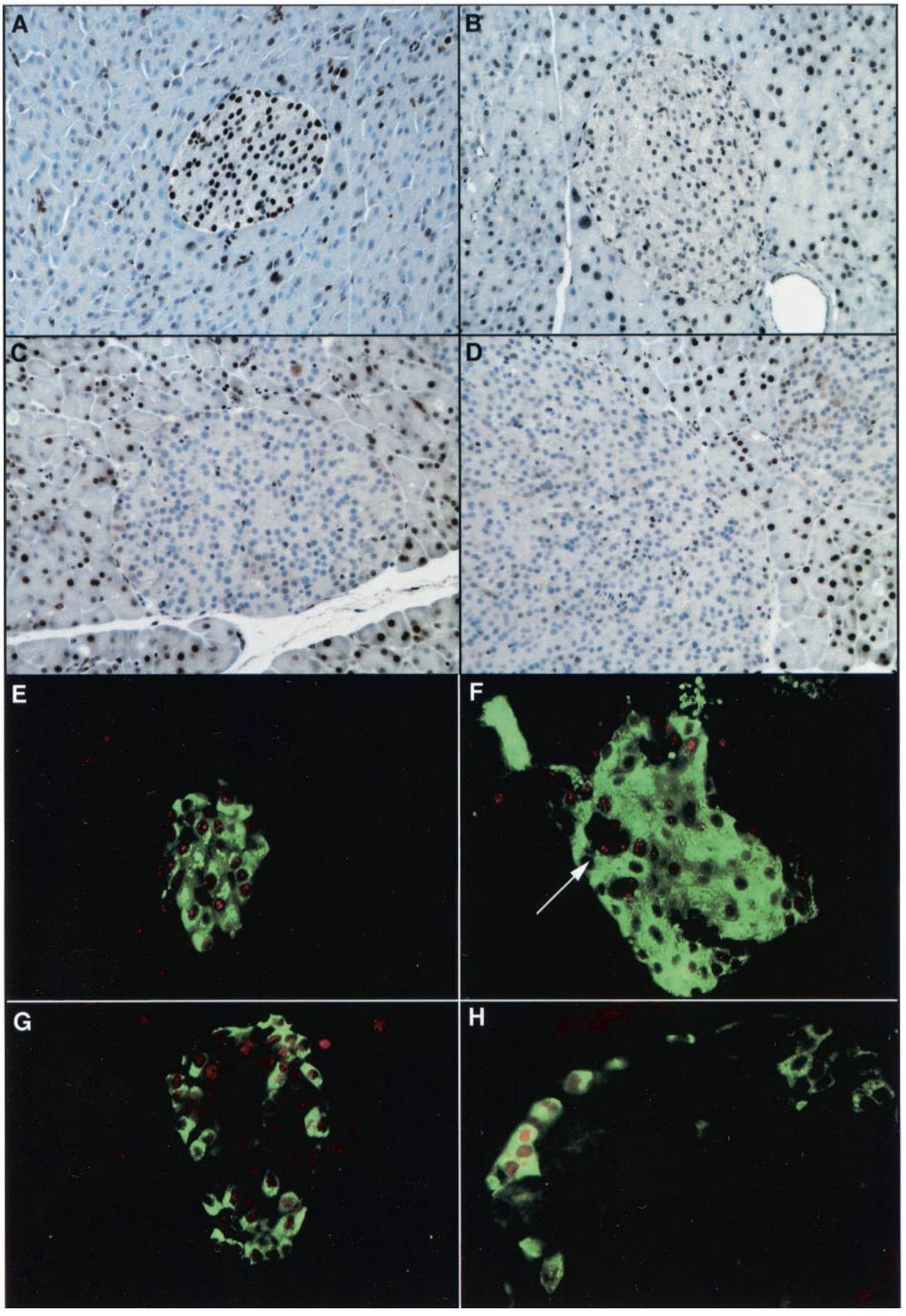
FIG. 3. Histology and tumor incidence. (A) Pancreatic islet morphology of 24-week-old $Men1^{\Delta N/\Delta N}$ mice with one of three RIP-cre transgenes. Sections were stained with hematoxylin and eosin and photographed at 100× magnification. (B) Tumor incidence in $Men1^{\Delta N/\Delta N}$; *RIP-cre* mice. Mice carrying the RIP2-cre (M) transgene develop tumors at an earlier age than either the RIP7-cre (H) or the RIP1-cre (E) mice, as evaluated by determining the percentage of mice with no tumor formation at the scheduled date of autopsy. (C) Tumor incidence in $Men1^{\Delta N/+}$; *RIP-cre* mice. Heterozygote floxed mice with a RIP-cre transgene develop tumors more slowly than the homozygotes. The rate is apparently independent of cre expression levels, as shown by the percentage of mice tumor free at the scheduled date of autopsy.

Cell proliferation and apoptosis. Cell proliferation in 6-month-old mice was evaluated with BrdU. Mice were injected intraperitoneally with a 200-mg/kg of body weight solution of BrdU in 0.8% saline at a dose of 0.1 ml/10 g of body weight. Tissue was harvested 1 h postinjection and fixed in 70% ethanol at room temperature. Tissue was embedded in paraffin and sectioned at 4 μm prior to staining with an anti-BrdU antibody followed by DAB detection. Apoptosis was evaluated by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays using the Apoptag peroxidase kit (Intergen Company, Purchase, N.Y.) in accordance with the manufacturer's instructions.

CGH. Genomic DNA from six pancreatic tumors and six pituitary tumors was extracted by following standard procedures. Hybridization was performed on karyotypically normal metaphase chromosomes (C57B/6 strain) with an excess of mouse Cot1 DNA (Gibco-BRL, Gaithersburg, Md.). DNA labeling, hybridization, and detection were performed as described previously (24). Images were acquired with a DMRXA epifluorescence microscope (Leica, Wetzlar, Germany) using fluorochrome-specific filters (Chroma Technologies, Brattleboro, Vt.). Quantitative fluorescence imaging and comparative genomic hybridization (CGH) analysis were performed with CW4000CGH software (Leica Microsystem Imaging Solutions, Cambridge, United Kingdom). Further details can be found at http://www.riedlab.nci.nih.gov/. Complete results of CGH analysis can be retrieved from the National Cancer Institute/National Center for Biotechnology Information SKY and CGH website at www.ncbi.nlm.nih.gov/sky/skyweb.cgi.

RESULTS

Generation of floxed *Men1* mice containing the cre transgene. Mice with floxed exons 3 to 8 of the *Men1* gene were generated by breeding the existing line $Men1^{TSM/+}$ (6) with the ubiquitously expressing cre line, EIIa-cre (20). Mice with only the neomycin cassette deleted (Fig. 1A) were bred to homozygosity and found to be fully viable and fertile, with offspring generated in expected Mendelian ratios. Homozygotes, termed $Men1^{\Delta N/\Delta N}$ mice, were then bred to one of three independently generated lines of transgenic mice expressing cre recombinase from the rat insulin promoter (RIP-cre). These three lines of RIP-cre mice vary in construction and site of genome integration (Fig. 1B): the RIP7-cre (or H) line contains 10 kb of the rat insulin I upstream promoter region to drive expression of cre recombinase, the RIP1-cre (or E) line contains 700 bp of the rat insulin I promoter region (3), and the RIP2-cre (or M) line contains 668 bp of the rat insulin II promoter. The RIP2-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cre construct also contained a nuclear localization signal, an optimized translation initiation codon (Kozak consensus), and a portion of the human growth hormone gene to facilitate splicing and expression of the cre transgene (28). Heterozygous mice carrying both the *Men1* ΔN allele and a RIP-cre transgene were then mated to generate homozygous *Men1*$^{\Delta N/\Delta N}$; *RIP-cre* animals. These animals were evaluated for up to 72 weeks, along with heterozygous and wild-type littermates, for hormone hypersecretion and tumor development.

Evaluation of cre expression in transgenic mice. All lines of RIP-cre transgenic mice were examined for tissue specificity and level of cre expression by being crossed with the Z/AP reporter mice [full strain designation is Tg(ACTB-Bgeo/ALPP)1Lbe] (23). The Z/AP mice contain the chicken beta actin promoter upstream of a floxed beta galactosidase gene, which is followed by the heat-stable human placental AP gene (Fig. 2A). This allows specific staining for lacZ and/or the heat-stable human placental AP to detect cre recombinase activity. The pancreatic islets of 8-week-old RIP7-cre mice showed the weakest cre recombination, estimated at approximately 10 to 20% of pancreatic beta cells, the RIP1-cre line exhibited a low-to-moderate recombination, approximately 20 to 30% of beta cells, and the RIP2-cre line had the highest recombination activity, approximately 80 to 90% of beta cells (Fig. 2B). By this analysis, no extraneous cre expression was detected in heart, liver, lung, spleen, kidney, adrenal, duodenum, stomach, seminal vesicles, testis, ovary, uterus, brain, parathyroid, or thyroid, although nonpancreatic cre expression in the RIP2-cre line has been reported (18). The RIP1-cre and RIP2-cre lines exhibited cre expression in the pituitary as well as in the pancreatic islets (data not shown). Pituitary tissue from the RIP7-cre line was not examined for cre expression.

Floxed *Men1* mice expressing RIP-cre develop pancreatic and pituitary lesions. *Men1*$^{\Delta N/\Delta N}$; *RIP-cre* mice were studied over a 72-week period, with animals autopsied at 6-week intervals to examine pathology of the pancreas and other organs. At 20 weeks of age, atypical hyperplastic islets (defined by abnormally large islets containing pleomorphic cells with variable size and shape) were observed in the *Men1*$^{\Delta N/\Delta N}$ mice carrying RIP7-cre or RIP1-cre transgenes (Fig. 3A). The earliest adenomas were detected at 44 weeks in the RIP7-cre line and at 30 weeks in the RIP1-cre line. In 60-week-old mice, islet volume in *Men1*$^{\Delta N/\Delta N}$; *RIP1-cre* mice was increased by sevenfold compared to that in age-matched littermates with only *RIP1-cre* (data not shown). Islet volume in *Men1*$^{\Delta N/\Delta N}$; *RIP7-cre* mice was not measured. Pancreatic islet lesions in *Men1*$^{\Delta N/\Delta N}$; *RIP2-cre* mice were more pronounced, with atypical hyperplastic islets appearing as early as 4 weeks; foci of adenoma developed within atypical islets by 20 to 28 weeks, and multiple adenomas developed as early as 23 weeks (Fig. 3A). Islet volume in 60-week-old *Men1*$^{\Delta N/\Delta N}$; *RIP2-cre* mice was increased 20- to 26-fold compared to that in age-matched littermates with only *RIP2-cre* (data not shown). No invasion of acinar tissue, progression to carcinoma, or extrapancreatic metastasis was observed in any of the cre-expressing mice.

Tumor incidence in the *Men1*$^{\Delta N/\Delta N}$; *RIP-cre* mice correlates with the levels of cre expression seen in the Z/AP crosses. As expected, floxed mice with RIP2-cre had an earlier age of tumor onset than those with the RIP7-cre and RIP1-cre transgenes (Fig. 3B). However, heterozygote floxed mice with RIP-cre transgenes developed pancreatic islet tumors later in life, with no obvious correlation to the cre expression level (Fig. 3C).

Pituitary adenomas developed in a subset of the animals with all three RIP-cre constructs and can be attributed to aberrant cre expression in the pituitary. Pituitary adenoma developed in 18% (6 of 33) of homozygous floxed mice with RIP7-cre, 12% (3 of 26) with RIP1-cre, and 56% (19 of 34) with RIP2-cre by the date of scheduled autopsy. The large pituitary tumors and associated mortality in aged homozygote floxed mice with RIP2-cre limited study of these animals beyond 56 weeks.

All pituitary adenomas were derived from the pars distalis, as judged from expression of prolactin (data not shown). Immunohistochemical staining for adrenocorticotropin was negative (data not shown). A striking sex bias was evident in the homozygous floxed, RIP2-cre mice, with 81% (13 of 16) of virgin female mice versus 33% (6 of 18) of male mice developing pituitary adenoma. Homozygous floxed RIP7-cre and RIP1-cre mice exhibited a milder sex bias, with pituitary adenomas developing in 25% (3 of 12) of virgin females versus 14% (3 of 21) of males and 15% (2 of 13) of virgin females versus 0.8% (1 of 13) of males, respectively.

Loss of menin in pancreatic lesions. Pancreatic islets in the *Men1*$^{\Delta N/\Delta N}$; RIP2-cre mice were evaluated for *Men1* status by IHC with C-terminal menin antibody SQV-R4 or SQV-R5. Compared to cells in wild-type and *Men1*$^{\Delta N/+}$ mice, a majority of the cells in the atypical hyperplastic islets and adenomas of aged *Men1*$^{\Delta N/\Delta N}$; *RIP2-cre* mice were not immunoreactive to menin antibodies (Fig. 4A to D). To demonstrate beta cell specificity and to determine if loss of menin in the beta cells of these islets results in immediate abnormalities, 4-week-old *Men1*$^{\Delta N/\Delta N}$; *RIP2-cre* mice were evaluated by dual immunofluorescence for menin and insulin and for menin and glucagon (Fig. 4E to H). As expected, menin and insulin were both present in the beta cells of the wild-type islets. In the *Men1*$^{\Delta N/\Delta N}$; *RIP2-cre* mice, menin expression was decreased or absent in most cells producing insulin. Additionally, cells in islets of *Men1*$^{\Delta N/\Delta N}$; *RIP2-cre* mice which stained strongly for menin were also strongly positive for glucagon, suggesting that

FIG. 4. Loss of menin in beta cells. (A to D) Menin IHC with the SQV-R4 antibody. Brown nuclear staining indicates the menin protein. (A) Menin IHC on the wild-type pancreas of a 56-week-old animal. (B) Pancreas of a 60-week-old *Men1*$^{\Delta N/+}$; *RIP2-cre* mouse showing a possible reduction (50%) of menin in the nuclei of a hyperplastic islet, but not complete loss. (C and D) Pancreas of a 48-week-old *Men1*$^{\Delta N/\Delta N}$; *RIP2-cre* mouse showing absence of menin in most of the nuclei of atypical islets (C) and tumors (D), while acinar tissue retains menin staining. (E to H) Dual immunofluorescence in beta cells. (E) Wild-type pancreatic islet stained for menin (red) and insulin (green). (F) Pancreas from a 4-week-old *Men1*$^{\Delta N/\Delta N}$; *RIP2-cre* mouse showing loss of menin in insulin-positive cells and presence of menin in cells negative for insulin (arrow). (G) Wild-type pancreas stained for menin (red) and glucagon (green). (H) Pancreas from a 4-week-old *Men1*$^{\Delta N/\Delta N}$; *RIP2-cre* mouse showing retention of menin in the cells positive for glucagon but its absence in the majority of the other islet cells.

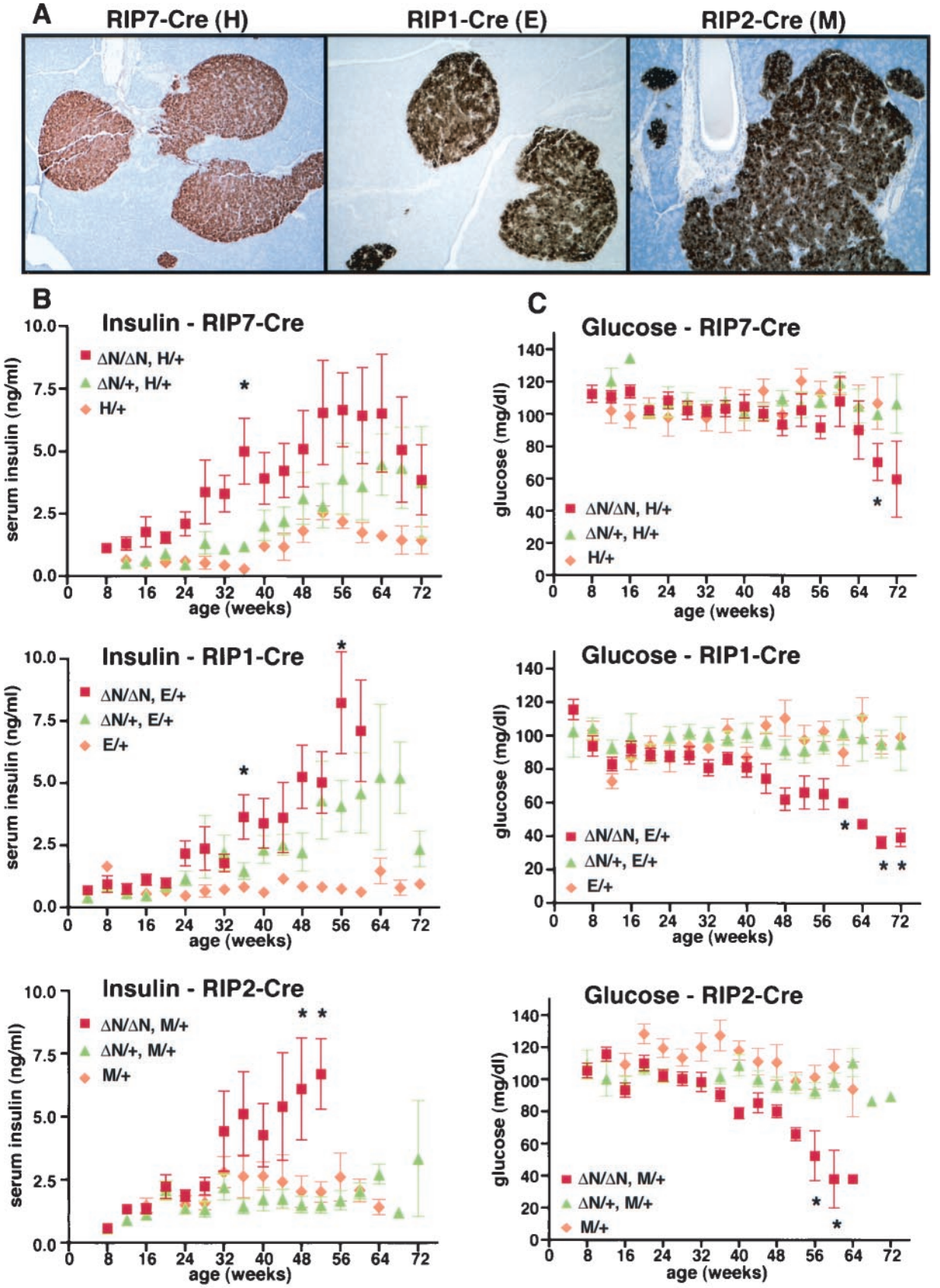

FIG. 5. Insulin IHC and blood chemistry. (A) Insulin IHC on sections from *Men1*$^{\Delta N/\Delta N}$; *RIP7-cre*/+ (H), *Men1*$^{\Delta N/\Delta N}$; *RIP1-cre*/+ (E), and *Men1*$^{\Delta N/\Delta N}$; *RIP2-cre*/+ (M) mice showing insulin production by atypical islets. (B and C) Serum insulin and blood glucose of fasted *Men1* floxed mice with the indicated cre transgene. Red squares, *Men1*$^{\Delta N/\Delta N}$; *RIP-cre* mice; green triangles, *Men1*$^{\Delta N/+}$; *RIP-cre* mice; gold diamonds, mice containing a cre transgene but wild-type for *Men1*. Error bars, standard error of the mean; asterisk, $P < 0.005$ by analysis of variance.

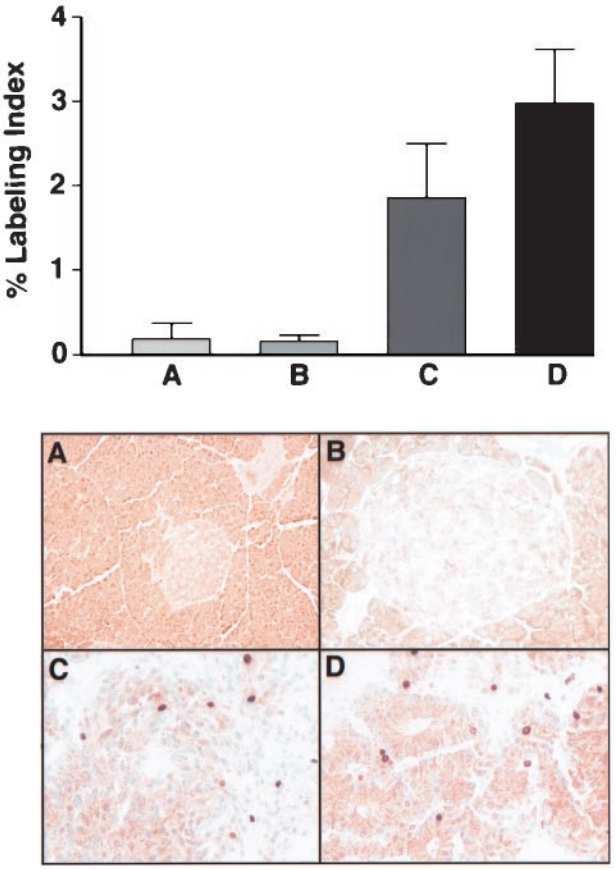

FIG. 6. Islet proliferation as shown by BrdU labeling. Islets that appear normal in both wild-type (A) and RIP2-cre-positive (B) control mice show minimal labeling, whereas atypical islets (C) and adenomas (D) show increased BrdU labeling. This suggests that increased cellular proliferation contributes to tumorigenesis.

menin-positive cells are mostly nonbeta cells. This result was further substantiated by dual immunofluorescence with menin and somatostatin, where menin staining was present in delta cells staining positive for somatostatin (data not shown).

Hormone hypersecretion in mice with atypical hyperplasia and tumors. Atypical hyperplastic islets and tumors in all mice expressed insulin, as evaluated by insulin IHC (Fig. 5A). These mice had elevated fasting serum insulin levels (Fig. 5B), but the modest level of elevation was most likely a reflection of an increase in the number of insulin-producing cells as opposed to increased insulin expression per cell. As expected, elevated serum insulin levels were accompanied by a decrease in fasting blood glucose (Fig. 5C), and all blood chemistry data correlated with level of cre expression and age of tumor onset.

Atypical hyperplasia and tumor formation are attributable to increased cellular proliferation. To examine the kinetics of cellular proliferation in the pancreatic islets, tissue was subjected to BrdU and TUNEL analyses to visualize cells in cycle and undergoing apoptosis, respectively. BrdU staining of 6-month-old mice clearly demonstrated an increase in labeling in the atypical hyperplastic and adenomatous lesions of the pancreas, whereas age-matched wild-type mice exhibited minimal BrdU labeling index, as expected, due to the low rate of pancreatic islet turnover in normal adult pancreas (Fig. 6A). Additionally, normal-size islets in 6-month-old mice containing a RIP-cre transgene but lacking the floxed menin alleles were similar to wild-type islets in having a very low labeling index (Fig. 6B). The elevated BrdU labeling demonstrates the increased cellular proliferation in the beta cells of atypical hyperplastic and tumorigenic islets (Fig. 6C and D). TUNEL assays on pancreatic tissue from these mice indicated no increase in apoptosis, and no apoptotic bodies were visualized histologically by hematoxylin and eosin staining (data not shown).

CGH. Tumors from *Men1*$^{\Delta N/\Delta N}$; *RIP2-cre* and heterozygous *Men1* knockout mice were analyzed by CGH to detect any chromosomal loss or duplication as a result of tumorigenesis. Pancreatic insulinomas had duplication of chromosome 11, whereas pituitary adenomas showed gain of chromosome 15, regardless of whether the tumors were derived from conditional or conventional knockout mice (Fig. 7). Tumors from heterozygous conventional knockout mice also had loss of chromosome 19, consistent with the loss of heterozygosity (LOH) at the *Men1* locus as previously described (6).

DISCUSSION

Capable of causing life-threatening hypoglycemia, insulinomas are rare and almost always benign. In MEN1, approximately 10% of affected individuals develop insulinomas, and these account for approximately one-third of the pancreatic lesions detected in MEN1 patients (the remainder secrete gastrin, somatostatin, or glucagon or are nonsecreting). Mouse models of human syndromes such as MEN1 offer unique opportunities to study disease progression and to test potential therapies.

Due to the embryonic lethality of the conventional homozygote *Men1* knockout mouse, a conditional knockout was generated to study insulinoma formation in the pancreata of mice lacking *Men1* in the islet beta cells. As early as 4 weeks of age, the pancreatic islet cells of homozygous conditional knockout mice appear subtly abnormal in morphology; by 23 weeks, multiple adenomas appear. The severity of pancreatic lesions in these mice correlates with the expression level of the cre recombinase, supporting the notion that these results are a direct consequence of *Men1* loss. As expected, *Men1*$^{\Delta N/+}$; *RIP-cre* mice do not develop pancreatic lesions until later in life than *Men1*$^{\Delta N/\Delta N}$; *RIP-cre* mice, presumably due to the length of time necessary to acquire random inactivation of the wild-type *Men1* allele.

All experimental mice in this study exhibited alterations in blood chemistry compared to littermate controls. The development of hyperplasia and tumors resulted in increased fasting serum insulin levels and decreased fasting blood glucose levels. The severity of hyperinsulinemia and hypoglycemia correlated with the level of cre expression (as evaluated by Z/AP staining). Judging by islet staining for insulin and the relatively mild increase in serum insulin (and corresponding decrease in blood glucose), insulin hypersecretion in *Men1*$^{\Delta N/\Delta N}$; *RIP-cre* mice likely reflects failure to adequately suppress insulin secretion in the setting of an increased number of insulin-secreting beta cells.

A secondary consequence of this conditional mouse model

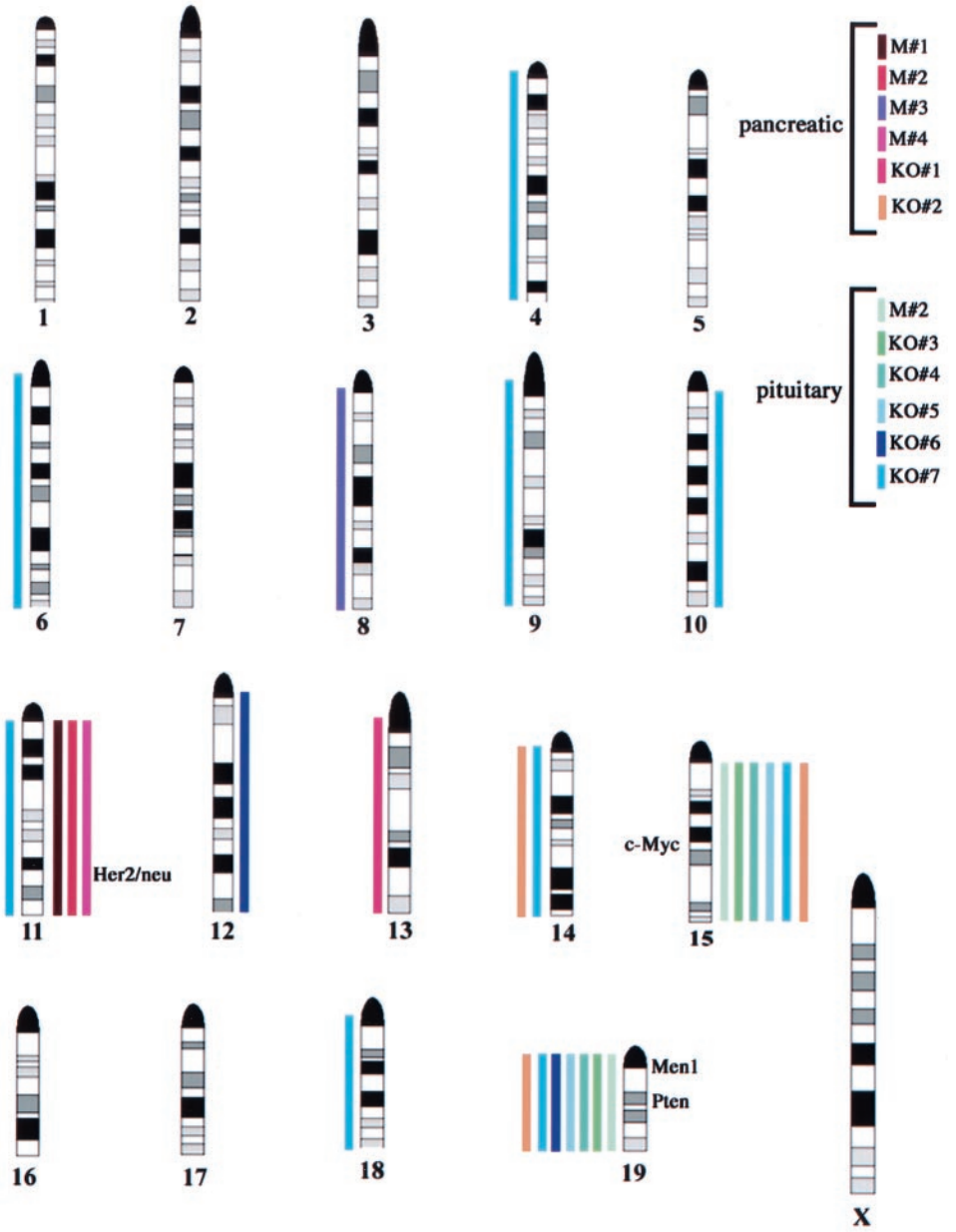

FIG. 7. CGH of menin null tumors. CGH was performed on tumor samples from four *Men1*$^{\Delta N/\Delta N}$; *RIP2-cre*/+ mice (M1 to M4) and seven conventional heterozygote knockout mice (KO1 to KO7). Pancreatic samples show gain of chromosome 11, whereas pituitary samples show duplication of chromosome 15, regardless of genotype. Conventional knockout samples have the expected loss of chromosome 19 and, thus, LOH for the Men1 locus. Bars to the left of chromosomes indicate chromosomal loss, and bars to the right indicate chromosomal gains.

system is the development of pituitary tumors. The pituitary adenomas are identical to the tumors that develop in the *Men1* conventional knockout, in that they are derived from the pars distalis, express prolactin, and develop preferentially in female mice. Although these tumors arise due to leaky expression of the RIP-cre transgenes, they still mimic the human phenotype in both histological appearance and function.

The pancreatic islets of conventional and conditional *Men1* knockout mice appear to progress through four distinct morphological stages of islet transformation (6). Islets in *Men1* conventional heterozygous knockout mice show a neoplastic progression from normal (stage 1) to large or hyperplastic (composed of an excessive number of apparently normal islet cells) (stage 2) to hyperplastic with focal atypia (composed of abnormally sized and shaped cells) (stage 3) to pancreatic islet tumors (frank adenoma) (stage 4). LOH analyses of DNA captured at these various stages of islet transformation revealed that the wild-type *Men1* allele is retained in hyperplastic islets (stage 2) and absent in tumors (stage 4) (6). It was hypothesized that the gradual enlargement of pancreatic islets occurred as a consequence of a dosage effect resulting from reduced amounts of menin protein (6). For homozygous con-

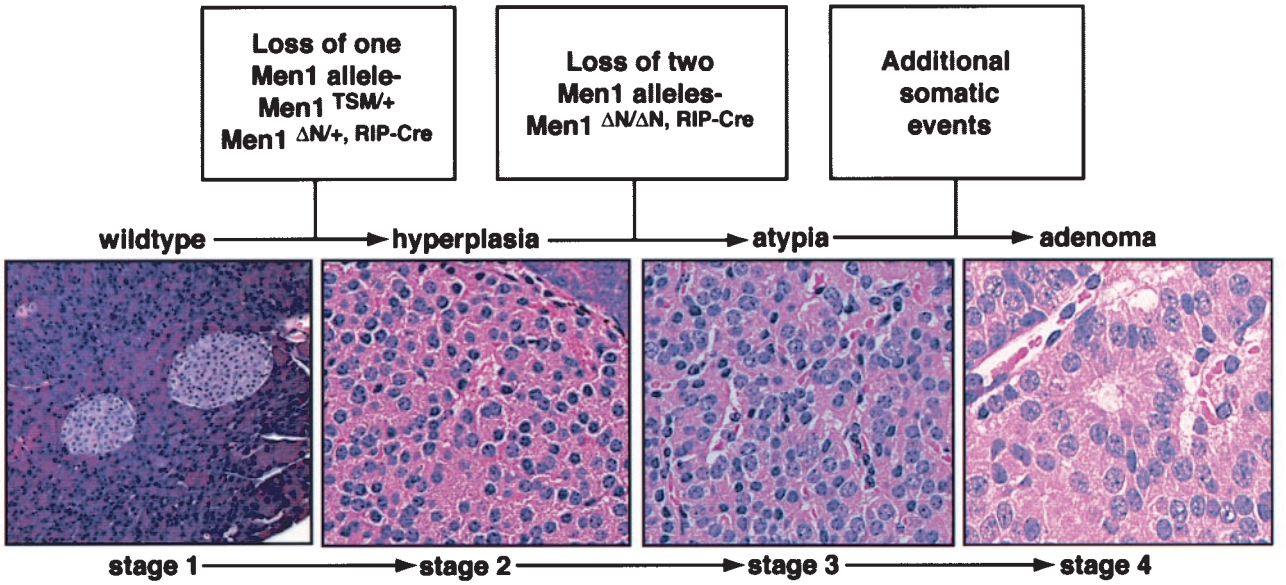

FIG. 8. Model of *Men1* function in islets. Stage 1 shows wild-type islets (10×). Stage 2 is a uniform population of hyperplastic islet cells (20×). Stage 3 is atypical islet hyperplasia, showing pleomorphism of hyperplastic islet cells (20×), and stage 4 shows the pseudorosette pattern typical of adenoma. Loss of one *Men1* allele, such as in the conventional heterozygote knockout or the heterozygote conditional mice with RIP-cre, leads to islet hyperplasia over time. Loss of the second allele, either by homozygous conditional targeting in the presence of RIP-cre or by LOH (for conventional heterozygote knockout of *Men1*), leads to atypia. Subsequent somatic events are required for tumor formation.

ditional knockout mice with high levels of RIP-cre expression (as in RIP2-cre mice), the vast majority of the islets appeared to be in stage 3 or stage 4 of islet transformation. IHC methods of menin detection, reported here for the first time, and immunofluorescence analysis confirmed loss of menin in the majority of beta cells of conditionally null islets and suggest that loss of menin in the beta cell causes an atypical islet phenotype. Atypical islets lacking menin are found at a high frequency in mice as young as 4 weeks, presumably because cre-mediated inactivation of both *Men1* alleles occurs at a very early age.

BrdU staining in *Men1*$^{\Delta N/\Delta N}$; *RIP-cre* mice indicated an increase in cellular proliferation in beta cell hyperplasias and adenoma, and TUNEL staining indicated no increase in apoptosis. However, since the apoptosis rate in wild-type beta cells is quite low (14), apoptosis could be suppressed in these cells by the additional gene inactivations required for adenoma formation, and the effect could go undetected. Given that menin expression is absent in most of the beta cells of young homozygotes but that frank tumorigenesis does not appear for 6 to 12 months, it is clear that adenoma formation is a multistep process, presumably involving one or more somatic events, which ultimately result in clonal expansion. Thus, homozygous loss of menin expression is necessary but not sufficient for adenoma formation. This is not unprecedented. For example, the RIP-Tag2 model (14) is a transgenic line in which the RIP1 promoter directs the expression of the simian virus 40 T-antigen (Tag), resulting in functional disruption of the retinoblastoma protein (Rb) and p53 pathways. These mice predictably develop hyperplasia by 4 to 5 weeks, angiogenic hyperplasia by 7 to 9 weeks, and adenoma or, less frequently, invasive carcinoma by 10 to 12 weeks. However, only a subset of islets develop these lesions, suggesting that, while Tag expression is fully penetrant and necessary for tumorigenesis in these mice, additional events are required. Support for this notion comes from the fact that multiple independent copy number abnormalities have been detected in RIP-Tag tumors by LOH, CGH, and array CGH analyses (8, 17, 26). A similar circumstance appears to apply in cells that are null for menin. Preliminary CGH data for *Men1*-deficient tumors indicate an increase in copy number of the entire mouse chromosome 11 in pancreatic insulinomas and mouse chromosome 15 in pituitary prolactinomas. Several oncogenes are present in these duplicated regions, including the c-Myc and ErbB2/Her2/Neu oncogenes, and further study is required to understand the mechanistic relevance of these chromosomal gains.

Therefore, based on a combination of supporting data collected from the conventional and conditional knockout mice, including menin IHC, LOH, and CGH, we propose the following model (Fig. 8). Loss of one *Men1* allele results in menin haploinsufficiency and islet hyperplasia over time (stage 2). The loss of two *Men1* alleles results in atypia (stage 3), with additional somatic mutations leading to tumor formation (stage 4).

The conditional *Men1* knockout mice represent a valuable model of beta cell hyperproliferation and can be utilized to study potential therapeutic interventions and to shed further light on the mechanism by which menin regulates cell growth. In that regard, islet hyperplasia is also a phenotype observed in mice expressing mutant cdk4 protein cdk4 R24C (29, 30), which is unresponsive to the inhibitory effects of p16ink4a and presumably other members of the ink4 family of cyclin-dependent kinase inhibitors. Cdk4 R24C homozygous mice express normal levels of the mutant protein, which properly hyperphosphorylates Rb for cell cycle regulation. These mice have

increased body weight (5 to 10% higher than wild-type littermates), which implies aberrant, unregulated growth in some tissues. One such tissue is the pancreatic islet, and mice expressing cdk4 R24C exhibit islet hyperplasia, with islet area 7 to 10 times that of wild-type littermates. Insulin IHC indicates that these hyperplasias, like the hyperplasias in the *Men1*$^{\Delta N/\Delta N}$; *RIP-cre* mice, are beta cell derived and produce insulin. cdk4 knockout animals (30), on the other hand, are significantly smaller than wild-type littermates and develop insulin- deficient diabetes at an early age. Islet volume for these mice is significantly decreased compared to that for wild-type littermates, and the expression levels of all beta cell-specific genes are also dramatically reduced. The phenotypes evident in the cdk4 knockout and the cdk4 R24C mutant mice expose a selective requirement for cdk4 function in beta cell proliferation and development via the Rb pathway. Further evidence suggesting a role for menin in the Rb pathway includes that from p18 p27 knockout mice (10). p18 $^{-/-}$ p27$^{+/-}$ mice develop islet hyperplasia in the pancreas in addition to many other types of endocrine tumors frequently observed in MEN syndromes, including pituitary adenoma, adrenal tumors, and parathyroid lesions. This is in contrast to the p18 and p27 null mice, which do not develop pancreatic lesions as individual gene knockouts. The functions of cdk4, p18, and p27 in cell cycle regulation via Rb may be key factors in understanding the tumor suppression role played by menin in the endocrine pancreas and perhaps other endocrine tissues. Crossbreeding these mutant mice may be a revealing approach.

In conclusion, the generation of a conditional mouse knockout of *Men1* provides new insight into the steps by which loss of this classic tumor suppressor gene leads to endocrine tumorigenesis in mice and men. The stage is now set to examine more closely the pathway in which menin participates in vivo and to test potential interventions that might benefit those with this important familial cancer syndrome.

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